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Sex Determination and Genome Size in *Catolaccus grandis* (Burks, 1954) (Hymenoptera: Pteromalidae)

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Abstract.—Complementary sex determination (CSD) is a common form of the haplodiploid sex determination system found in all wasps, ants, and bees (Hymenoptera). Exceptions exist to CSD, but too few have been documented to make phylogenetic conclusions. Males that are homozygous at CSD loci are diploid and often sterile. Any effect that increases homozygosity (inbreeding and small population size) should increase the proportion of diploid males. We use flow cytometry to determine the genome size of males and females of the parasitic wasp *Catolaccus grandis* (Burks) (Hymenoptera: Pteromalidae) (1 C = 455.4 ± 3.4 mb). We then score haploid and diploid males (and females) from populations that were 25% and 50% inbred. None of the 314 males scored were diploid. We conclude that the CSD system is very unlikely to exist in this species and discuss the implications for sex determination systems in the Pteromalidae and other chalcidoids.

Evolving independently at least eight and possibly as many as 15 times in mites and insects (White 1973, Mable and Otto 1998) in phylogenic lineages that represent nearly 20% of all animal species (Bell 1982, Bull 1983), haplodiploidy is the sole reproductive mode in Hymenoptera, including the pteromalid wasp *Catolaccus grandis* (Burks, 1954) (Hymenoptera: Pteromalidae) studied here. The most common system of haplodiploidy is arrhenotokous reproduction, where females develop from fertilized eggs and males from unfertilized eggs. The most studied form of arrhenotoky is the complementary sex determination (CSD) mechanism, whereby a sex locus with multiple alleles determines the sexual development of the offspring (Whiting 1943, Beye et al. 2003). In the CSD system, zygotes heterozygous at the CSD locus develop into biparental diploid females; males are produced as uniparental haploids that have one CSD allele. Uniparental males are typically produced as unfertilized eggs, but in some cases are produced as zygotes in which one parental genome is excluded post fertilization (Keller et al. 2001, Beukeboom et al. 2007).

The CSD form of arrhenotoky fails when the female is fertilized by a male whose single allele at the CSD locus is identical to one of the two CSD alleles that she carries. When this happens, half of her diploid fertilized progeny (all of whom should develop as female) are homozygous for a CSD allele and develop into biparental diploid males. Diploid males were first observed in a single locus CSD (sl-CSD) system by Whiting in *Bracon hebetor* Say, 1836 (Whiting 1943) but have been observed in very many species since (Trent et al. 2006). Diploid males are often sterile, yet lacking the division of chromosomes required to produce haploid sperm, may

produce diploid sperm and consequently, sterile triploid females (Whiting 1943, Stouthamer et al. 1992, Cowan and Stahlhut 2004). Whether in natural populations or in laboratory reared colonies, diploid males and triploid sterile females represent a reproductive cost, affecting fertility and sex ratio (Stouthamer et al. 1992, Wu et al. 2005, see exception in Cowan and Stahlhut 2004). Production of diploid males and triploid females is most apparent when the number of CSD alleles is reduced by population bottlenecks and founder events, or when homozygosity increases as a result of consanguinity. The latter is important, since inbreeding by sib-mating occurs often in hymenopteran species that are solitary or are social parasites (Schrempf et al. 2006).

The parasitoid pteromalid wasp, *C. grandis*, is economically important for use in bio-control of the boll weevil, *Anthonomous grandis* (Boheman, 1843) (Coleoptera: Curculionidae), and has been released experimentally for crop management in many areas of the southern U.S.A., including the Rio Grande Valley, Texas, San Angelo, Texas, and Aliceville, Alabama (Morales-Ramos et al. 1994, 1995a, 1998, Summy et al. 1995, 1997). An ectoparasitoid, *C. grandis* lays its eggs on boll weevil larvae (most often the third instar or pupa), greatly suppressing the numbers of the boll weevil and thereby reducing the damage caused to cotton (Summy et al. 1995). As an idiobiont, the female stops host development with a paralytic venom (Morales-Ramos et al. 1995b) and the emerging larva consumes its paralyzed host. These characteristics may give an advantage as an efficient biocontrol agent (Morales-Ramos et al. 1995b). However, production efficiency in insect mass rearing of *C. grandis* and related species can be problematic if sl-CSD is present, because that would necessitate a large number of sex alleles to minimize diploid male production. Special measures would need to be taken in colony management to avoid founder events, population bottlenecks, and inbreeding leading to a loss of the sex alleles.

Economically important hymenopterans are among the best studied, and their sex determination is among the best documented. The sl-CSD system is well demonstrated in the red imported fire ant (*Solenopsis invicta* Buren, 1972) and the honey bee *Apis mellifera* L., 1758 (Hymenoptera: Apidae); 11 to 19 alleles were determined for the complementary sex determiner (csd) locus in honey bees (Beye et al. 2003), with a smaller number of alleles (8 to 13) in the population of red imported fire ants, that went through a bottleneck when introduced into southern United States (Ross et al. 1993). The occurrence of CSD in the Hymenoptera has been demonstrated in symphytan, ichneumonid, and braconid species, but to date not in chalcidoids (Stouthamer et al. 1992). In several of these groups apparent exceptions exist, specifically in instances where inbreeding has not exposed sl-CSD (Wu et al. 2005).

The relative phylogenetic position of pteromalids within the tree of life is largely unknown, although studies are underway to remedy this situation (Castro and Dowton 2005, J. Heraty personal communication). Understanding the kind of sex determination system and the ancestral or derived state of the CSD system should be part of this phylogenetic effort. We assume that CSD is the ancestral mode of reproduction. There is little published research to prove this, however, especially in the more ancestral taxa (Cook and Crozier 1995). Here we test the hypothesis that a form of the CSD system exists in *C. grandis*. This represents the second test of CSD in Pteromalidae. We employ a new approach in studying CSD by determining genome size first, and then directly scoring males of consanguineous matings, using flow cytometry to score ploidy level. If a CSD system exists in this species, diploid male production, to the extent they survive, will be directly proportional to the level of consanguinity.

METHODS

Live Material

Catolaccus grandis from wild-caught individuals were used to initiate a reared population. The lab colony used here was founded with: 6 genomes from El Salvador, Central America (host plant of boll weevil: wild and cultivated cotton), 56 genomes from Tabasco, México (host plant of boll weevil: *Hampea nutricia* Fryxell (Malvaceae)), and 15 genomes from Oaxaca, México (host plant of boll weevil: *Cienfuegosia rosei* Fryxell (Malvaceae)). All *C. grandis* in this study were from controlled matings among reared individuals.

Crosses

A. Sib-mating offspring.—Virgin *C. grandis* females were mated with a single male. Their offspring were confined in individual Petri dishes and allowed to mate (sibling matings). Each female was isolated in a Petri dish 3–4 days after emergence for independent offspring evaluation. Host boll weevil larvae were presented to the *C. grandis* females encapsulated in Parafilm® using the method described by Cate (1987). Twelve Parafilm® encapsulated boll weevil larvae per female per day over five days were provided as hosts. Females that produced only male offspring were discarded (presumably they were not fertilized and had no opportunity to produce diploid males). Male offspring of fertilized females were prepared for flow cytometry.

B. Backcross offspring.—Virgin sibling couples were isolated in Petri dishes, allowed to mate, and were removed after offspring emergence. Families with only male offspring or without father survival were discarded. Daughters were paired with their fathers to induce mating and allowed to reproduce. Again, offspring with only males were not included in the analysis.

Flow Cytometry

Individual *C. grandis* males were prepared for flow cytometric analysis of genome size and ploidy level following Johnston et al. (2004). The head and thorax of a *C. grandis* was placed in a 1.5 ml Kontes Dounce tissue grinder into 1 ml of cold Galbraith buffer (per litre: 4.26 g $MgCl_2$, 8.84 g sodium citrate, 4.2 g 3-[N-morpholino] propane sulfonic acid, 1 mL Triton X-100, 20 µg/mL boiled Ribonuclease A, pH 7.2; Galbraith et al. 1983). Chicken red blood cells (CRBCs) were added to act as standards (1 C = 1212 Mb; Bennett et al. 2003). To release and isolate nuclei, the head and thorax plus the CRBCs in buffer solution were stroked 15 times with an A pestle and filtered through a 20-µm nylon filter. Propidium iodide, an intercalating dye that binds stoichiometrically to DNA and fluoresces in direct proportion to the amount of DNA present, was added to a final concentration of 50 ppm, and the mixture co-stained in the dark at 4°C for 20–40 minutes. The mean fluorescence of co-stained nuclei in replicate samples of each sex was quantified using a Coulter Epics Elite (Coulter Electronics, Hialeah, FL) with a laser tuned at 514 nm and 300 mW. Individual nuclei separate and pass across the exciting light source in the flow cell of the cytometry, where the nuclei are counted and the fluorescent light emitted from each nuclei is collected and quantified after passing a long pass filter to eliminate any reflected laser light. To avoid counting debris or nuclei with associated cytoplasmic tags, counting was activated by PI fluorescence, and only clean, singlet nuclei with low light scatter were included in the analysis. Genome size was estimated for each male and female *C. grandis* by first calculating the ratio of the fluorescent intensity of *C. grandis* nuclei to that of the CRBCs standard. The genome size was then determined by multiplying this ratio by the amount of DNA in the CRBCs.

Analysis

The genome size of *C. grandis* is given as the average (+/− standard error) of the

estimated genomes. A minimal estimate of the likelihood of scoring a diploid male is based on a binomial distribution, where the expectation of a diploid male is equal to the probability of homozygosity, assuming sufficient numbers of alleles at independent CSD loci that inbreeding is the only source of homozygosity.

RESULTS AND DISCUSSION

The genome size of *C. grandis* was estimated using a chicken red blood cell (CRBC) (1 C = 1212 Mb) standard and measured 1 C = 455.4 ± 3.4 Mb (Fig. 1 A, B). As expected, the haploid genome of males was half that of the diploid genome of females. The *C. grandis* genome is 1.37 times larger than that of the only other pteromalid genome scored to date, *N. vitripennis* (Walker) (1 C = 332.5) and 3 times larger than the genome of the only scored braconid wasp, *Habrobracon juglandis* (Ashmead, 1889) (1 C = 156.5) (Rasch et al.1975).

Catolaccus grandis were easily scored for ploidy level. Females revealed only a diploid peak (Figure 1A). Haploid males showed 2 fluorescent peaks with equal numbers of haploid and diploid (endoreduplicated) nuclei (Fig. 1 B). This endoreduplicated peak is observed because of the diploid tissue found in the muscle (Johnston et al. 2004). Diploid males (if present) would have shown only one strong diploid peak, as observed in the females. All 124 males from the F-2 gave a strong haploid peak as seen in Figure 2; no diploid males were scored. These results indicate that a CSD system, if it exists, is based on a number of loci in a multilocus complementary sex determination system (ml-CSD). A second set of inbred backcross offspring was produced, collected, and scored in order to estimate more precisely the number of genes involved in a potential ml-CSD system. Backcross offspring were selected because the expected 50% homozygosity in the backcross to the haploid male parent reduces the sample size needed to detect diploid males (Table 1). All males produced from the backcrosses (190 in total) were haploid.

If the CSD system exists for *C. grandis*, inbreeding should increase homozygosity and result in significant diploid male production. Given haplodiploid sex determination, sib mated and backcross zygotes had a probability of homozygosity at a single locus of 0.25 and 0.50 respectively. The failure to observe diploid males among the 314 scored males indicates that a CSD system, if it occurs at all in *C. grandis*, involves >5 independent loci. The probability that diploid males will be produced in significant numbers due to sib mating in nature, or due to small effective size in mass rearing colonies is low.

A possible source of error in these analyses is cannibalism or other forms of reduced survival of diploid males. Selective cannibalism based on semiochemicals is known to occur in honey bees, where the workers will consume any diploid males (Woyke 1963). A parasitoid of the codling moth, *Liotryphon caudatus* (Ratzeburg, 1848) (Hymenoptera: Ichneumonidae) with known cannibalism produces a significant diploid male population (T. R. Unruh, personal communication). This suggests, that in some haplodiploid species, cannibalism does not necessarily lead to selective killing of diploid males. In culture, *C. grandis* females lay eggs either on the boll weevil larvae or on the interior surface of the Parafilm® capsule where the host is presented as Parafilm® encapsulated larvae. In the rearing conditions here, the female is expected to lay 1, 2, or 3 eggs per host. An emerged *C. grandis* larva will move around and eat any other eggs or larvae that it encounters (Morales-Ramos and Cate 1992), and rarely are two parasitoid pupae found in the same host.

Two sources of data suggest that diploid male mortality cannot explain the absence of diploid males in our study. Fully 44.4% of hosts receive a single egg, and the

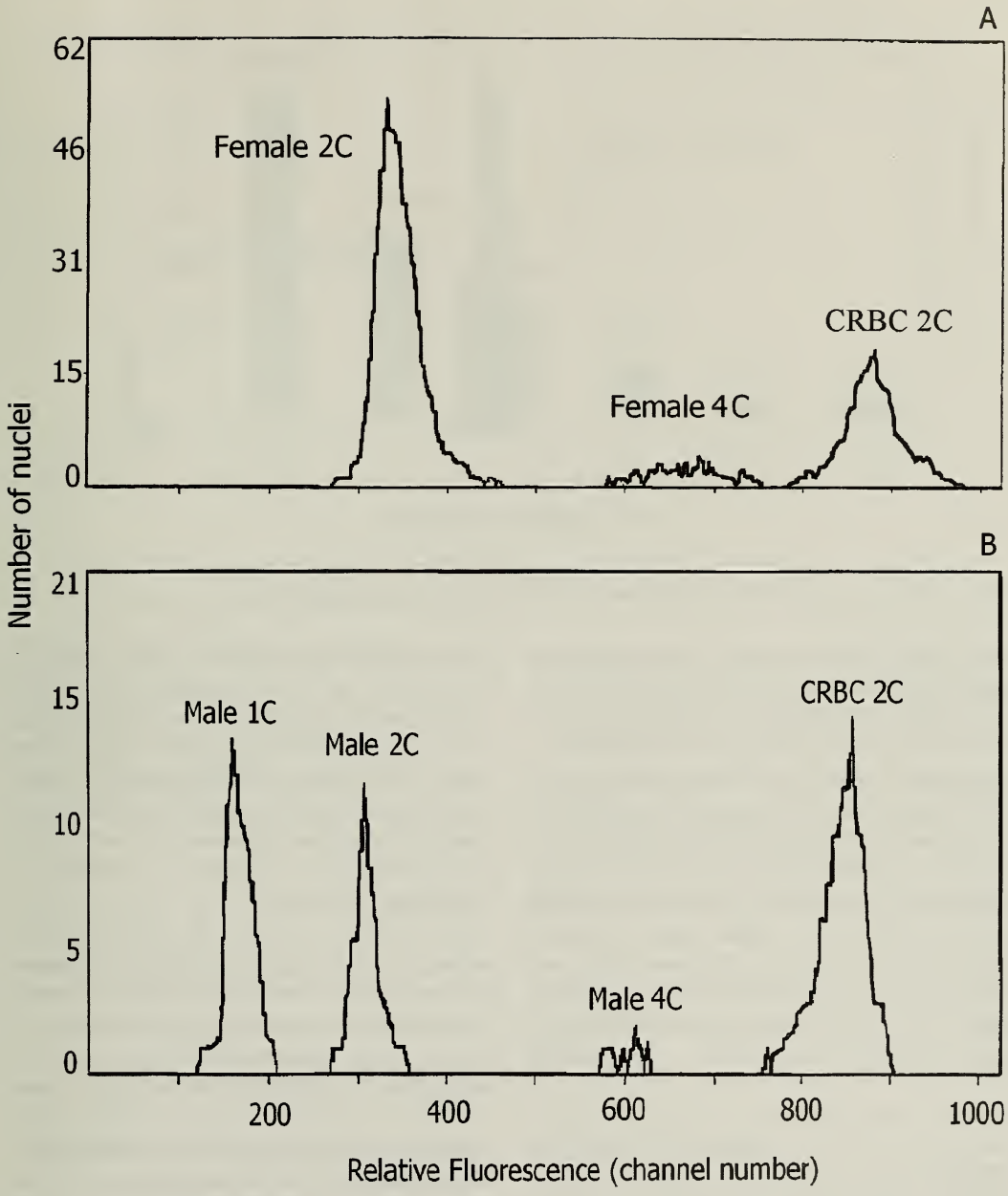

Fig. 1. Relative DNA staining in nuclei from the head of A) female *Catolaccus grandis* plus chicken red blood cells (CRBCs) and B) haploid male *C. grandis* plus CRBCs. Neural tissue produces the first 1 C peak in haploid males. DNA in the nuclei of muscle is largely endoreduplicated in haploid males (Johnston *et al.*, 2004) resulting in the diagnostic 1 C, 2 C and small 4 C peaks shown here. Relative DNA amount is calculated as the product of the ratio of the mean fluorescence of the leftmost peak (1 C in haploid males, 2 C in females)/mean fluorescence of CRBC standard multiplied by the estimated genome size of the CRBC standard (1140 Mb; Bennett et al. 2003).

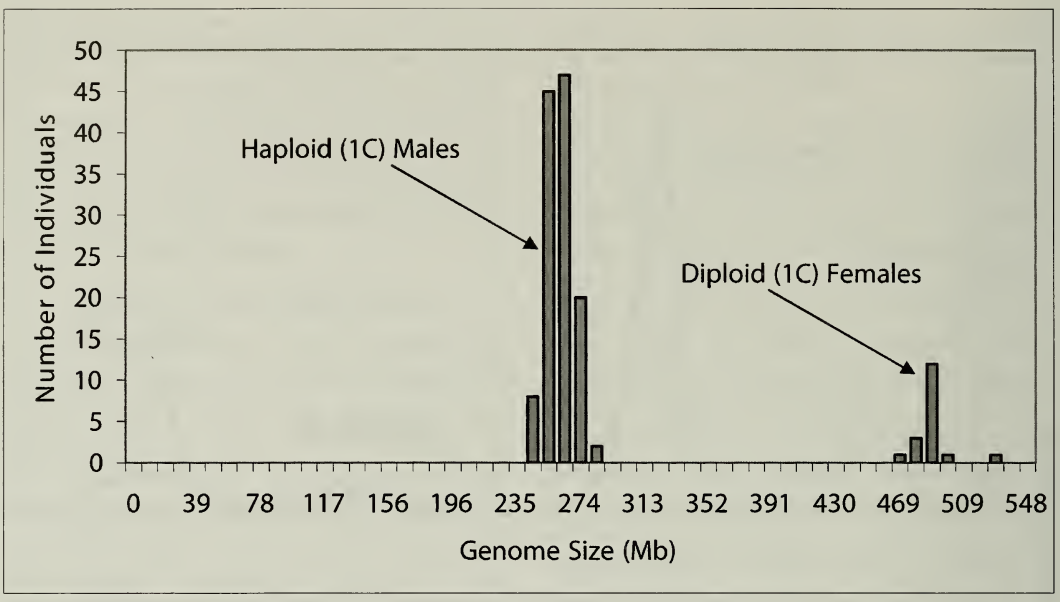

Fig. 2. Relative genome sizes of individuals of haploid males and diploid females.

remainder (55.6%) receive two or more eggs (Fig. 3). Even under the assumption that diploid males from single egg hosts are the only ones that survive cannibalism, a significant number of diploid males in a CSD system would be expected. Additional evidence that diploid males should have survived cannibalism is supported by the observation that there was no deviation of the sex ratio from what was expected. Under lab conditions, the sex ratio observed for the sib-matings was approximately 2.8:1. The previously published sex ratio in natural populations is 3:1 or 4:1 (Morales-Ramos and Cate 1992). A reduction in sex ratio (more surviving males) (1.2:1) was observed in the backcrosses, but this could be related to the age of the father. Gomez et al. (1997) exposed a virgin female every day during the life span of *C. grandis* males and observed a reduction over time in the ability of males to inseminate females.

Table 1. Sample size needed to detect a diploid male in F2 and backcross offspring (BC), given a sex determining system with one to five genes involved (ml-CSD) and a 4:1 female: male sex ratio in fertilized females under random mating based on binomial distribution.

	F2		BC	
Number of loci	P=95%	P=99%	P=95%	P=99%
1	10	13	4	6
2	18	27	6	9
3	66	101	10	15
4	258	396	18	27
5	1025	1575	35	76

Inbreeding, because it increases the chance of homozygosity and thus increases the appearance of diploid males, reduces the fitness of species with sl-CSD. In species with proven CSD, the effect of population bottlenecks and founder events that decrease the number of alleles and increase inbreeding may be reduced by modification of the CSD system, including inbreeding avoidance behaviors, and the development of a non-CSD system (Cook and Crozier 1995, Dobson and Tanouye 1998). One modification of CSD is to involve multiple loci, although the sex determination mechanism remains the same: females are heterozygous at one or more loci, while full fertility is expected only in haploid males where each CSD allele is present as a single copy (Paxton et

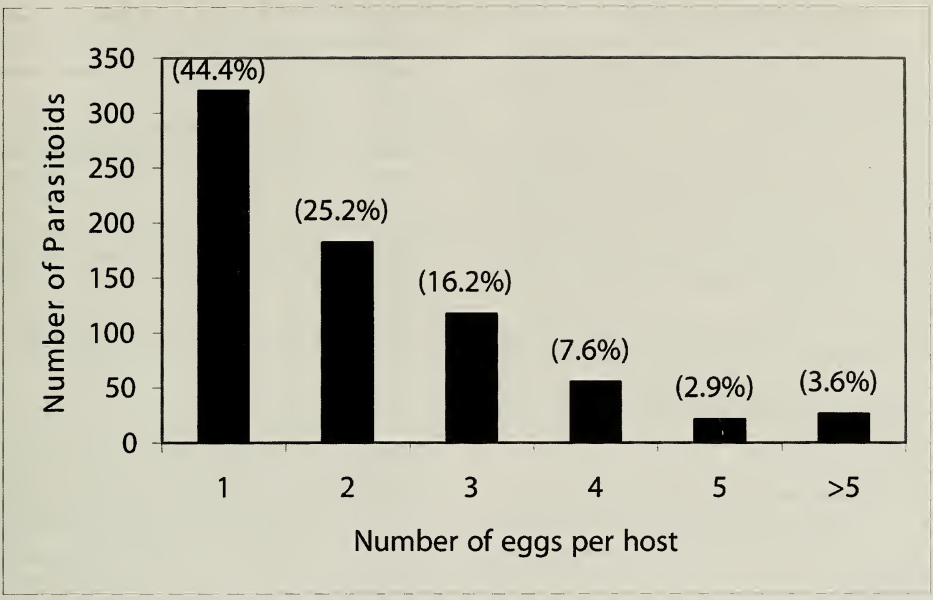

Fig. 3. The number and percentage (in parenthesis) of 721 hosts parasitized by 1, 2, 3, 4 and 5 or more eggs.

al. 2000). In ml-CSD, the chance of a homozygous diploid male is greatly reduced, even in inbreeding populations. Inbreeding effects have not been well tested experimentally for many Hymenoptera, however, and it can be argued that ml-CSD has not been persuasively demonstrated (Beukeboom et al. 2007), leaving the as best-known mechanism for inbreeding avoidance in the presence of CSD, the premating dispersal observed in *Bracon hebetor* (Ode et al. 1995). Because we experimentally controlled matings, mechanisms to avoid inbreeding were not an issue, and even an ml-CSD system is unlikely.

Single locus CSD has been demonstrated for at least 60 species of hymenopterans (Beukeboom et al. 2007), including most social species that have been studied (see exception in Schrempf et al. 2006). However, the CSD system is not the only mechanism for arrhenotokous reproduction. The absence of sl-CSD system has been conclusively demonstrated in *Nasonia vitripennis* (Walker, 1836) (Hymenoptera: Chalcidoidea) and the parasitoid *Heterospilus prosopidis* Viereck, 1910 (Hymenoptera: Braconidae) (Wu et al. 2005). It is also absent in the more distantly related ant *Cardiocondyla obscurior* Wheeler, 1929 (Formicidae), which shows no evidence of diploid males but does show inbreeding depression (Schrempf et al. 2006). Proposed alternative sex determination mechanisms in arrhenotokous insects include genomic imprinting, fertilization sex determination, genic balance sex determination, and maternal effect sex determination, which were all tested in populations of *N. vitripennis* (Dobson and Tanouye 1998, Beukeboom et al. 2007). The genomic imprinting model fits well for *N. vitripennis* (Trent et al. 2006) and may also be present in related Chalcidoidea.

Flow cytometry allowed us to quickly score for diploid male production, and we showed that the CSD model is unlikely to be the sex determination mechanism for the pteromalid, *C. grandis*. These results, along with fairly compelling evidence to reject CSD in one Trichogrammatidae (Chalcidoidea) species (Stouthamer and Kazmer 1994), raise the question of whether the CSD model arose independently or in a common ancestor of these species.

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